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Isolation, molecular characterization and *in vitro* differentiation of bovine Wharton's jelly-derived multipotent mesenchymal cells

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ABSTRACT

Extra-fetal tissues are a non-controversial and inexhaustible source of mesenchymal stem cells (MSCs) that can be harvested non-invasively at low cost. In the veterinary field, as in man, stem cells derived from extra-fetal tissues express plasticity, reduced immunogenicity, and have high anti-inflammatory potential making them promising candidates for treatment of many diseases. Umbilical cord mesenchymal cells have been isolated and characterized in different species, and have recently been investigated as potential candidates in regenerative medicine. In this study, cells derived from bovine Wharton's jelly (WJ) were isolated for the first time by enzymatic methods, frozen/thawed, cultivated for at least ten passages and characterized. Wharton's jelly (WJ)-derived cells readily attached to plastic culture dishes displaying typical fibroblast-like morphology and, although their proliferative capacity decreased to the seventh passage, these cells showed a mean doubling time of 34.55 ± 6.33 hours and a mean frequency of 1 colony forming unit fibroblast-like (CFU-F) for every 221.68 plated cells. The results of molecular biology studies and flow cytometry

analyses revealed that WJ-derived cells showed the typical antigen profile of MSCs and were positive for CD29, CD44, CD105, CD166, Oct-4 and c-Myc. They were negative for CD34 and CD14. Remarkably, WJ-derived cells showed differentiation ability. After culture in induced media, WJ-derived cells were able to differentiate into osteogenic, adipogenic, chondrogenic and neurogenic lines as shown by positive staining and expression of specific markers. On PCR analysis, these cells were negative for *MHC-II* and positive for *MHC-I*, thus reinforcing the role of extra-fetal tissue as an allogenic source for bovine cell-based therapies. These results provide evidence that bovine WJ-derived cells may have the potential to differentiate to repair damaged tissues and reinforce the importance of extra-fetal tissues as stem cell sources in veterinary regenerative medicine. A more detailed evaluation of their immunological properties is necessary to better understand their potential role in cellular therapy.

Key words: bovine, Wharton's jelly, stromal cells, characterization

1. INTRODUCTION

Increasing interest in veterinary stem cell therapy has led to research into new stem cell sources that can supply sufficient numbers of cells whilst minimizing risks to donors and recipients [1]. Extra-fetal stem cells are being investigated for this purpose in large animals [2]. Extra-fetal stem cells have been isolated from umbilical cord blood, amniotic fluid, amniotic membrane, umbilical cord matrix, yolk sac and placenta [3-9]. It has been shown that they possess properties of mesenchymal stem cells, and their application in regenerative medicine has increased over the past few years [10]. These studies show that umbilical cord, previously considered as a biomedical waste, is a source of stem cells with promising therapeutic applications in man as well as in livestock species.

The umbilical cord provides an inexhaustible source of stem cells and presents few, if any, ethical concerns. Indeed, umbilical cord can be collected after parturition since it is considered a waste tissue. In addition, the process of obtaining the cord tissue is relatively simple and non- invasive.

Mesenchymal stem cells (MSCs) have been isolated from different compartments of the umbilical cord in different species. In particular, they were obtained invasively, by a surgical intrauterine approach, from umbilical cord blood of sheep [11,12], non-invasively at the time of birth in the horse [13], in cattle, after caesarean section delivery [14], and from canine and feline fetuses at birth [15,16]. In 2006, for the first time in veterinary medicine, MSCs from cordon matrix, called Wharton's jelly (WJ), were obtained from porcine umbilical cord [17]. Wharton's jelly is the embryonic mucous connective tissue lying between the amniotic epithelium and the umbilical vessels [18]. It encloses the yolk sac, which is the source of primordial germ cells and the first hematopoietic stem cells [18]. Extra-embryonic tissues, which originate from the hypoblast and the trophectoderm, do not participate in gastrulation, therefore they are less differentiated than adult somatic tissues such as bone marrow or adipose tissue [2]. Wharton's jelly is a rich source of MSCs [19-21]. Recently, primitive MSCs were isolated from the umbilical cord matrix in caprine [22,23], canine [24] and equine species [4,13,25]. These cells show specific markers of pluripotency and MSCs markers and are able to differentiate into adipogenic, chondrogenic, osteogenic and neurogenic tissues [2]. The similarities in growth kinetics and expression of markers of pluripotency indicate their close resemblance to embryonic stem cells [26]. These markers, found in mouse and human embryonic stem cells, include the POU (Pit/Oct/Unc) domain-containing protein Oct-4, Sox-2 and Nanog. Some authors have shown that human umbilical cord matrix expresses low levels of transcription factors that play a central role in the regulation of pluripotency and self-renewal [18]. In contrast however, Carlin et al. [17] showed that cells derived from porcine umbilical cord matrix express markers of embryonic lineage Oct-3/4, Sox2 and Nanog. Contrary to observations in adult MSCs, WJ-MSCs share properties unique to fetal-derived MSCs, such as more rapid proliferation and greater *ex vivo* expansion capabilities [18,27]. Moreover, they have high potential to be differentiated *in vitro* [28], they express HLA-class I surface markers but do not express HLA-class II markers [29], and they are immunosuppressive in mixed lymphocyte assays by inhibition of T-cell proliferation [30,31]. For these reasons, these cells have raised interest for their potential uses in

cell and gene therapy, cloning, virological and biotechnological studies [32].

Despite the importance of bovine species as models for *in vivo* studies, little it is known about bovine MSCs. So far, they have been derived from umbilical cord blood [14], bone marrow [33,34] and amniotic membrane or amniotic fluid [8]. To date, only one paper on isolation and characterization of MSCs from bovine umbilical cord matrix [32] had been published but these cells were isolated by migration techniques and not by classical enzymatic digestion. The mechanical and enzymatic disaggregation of the tissue avoids problems of selection by migration, but perhaps more importantly, yields a higher number of cells more representative of the whole tissue in a shorter time. However, as well as the primary explant technique selecting on the basis of cell migration, the dissociation technique selects cells resistant to disaggregation but still capable of attachment [35].

The isolation of bovine MSCs from fetal adnexa is an interesting prospect because of the potential for these cells to be used for biotechnological applications. For the first time, we isolated, by enzymatic methods, presumptive MSCs from bovine WJ and were able to characterize them in terms of morphology, specific mesenchymal or pluripotent markers, proliferative and differentiation potential.

2. MATERIALS AND METHODS

2.1 Materials

Chemicals were obtained from Sigma Chemical (Milan, Italy) and tissue culture plastic dishes from Euroclone (Milan, Italy) unless otherwise specified.

2.2 Tissue collection

This study was approved by the Ethical Committee of the University of Milan and written owner consent was given. All procedures were conducted following standard veterinary practice and in accordance with 2010/63 EU directive on animal protection and Italian Law (D.L. No. 116/1992).

Fresh bovine umbilical cords were obtained after full-term births.

Bovine umbilical cords (n=3) were obtained from three cows following normal term pregnancies with spontaneous parturition in accordance with veterinary practice standard. Before the cows stood up breaking the cord, a surgical tape was placed at the calf junction and a second tie was tightened at approximately 30/40 cm from the first. The tie-limited cord portion was cut away with scissors. The harvested segment of the cord was washed twice in sterile saline solution and kept at 4°C in saline solution supplemented with 4 µg/mL amphotericin, 100 UI/mL penicillin and 100 µg/mL streptomycin and processed within 12 hours of collection.

2.3 Isolation and culture of WJ-derived cells

At the laboratory, the loose amnion was removed from the exterior of the cords and the cords were incised longitudinally to expose and remove umbilical vessels (arteries and veins). The remaining WJ-containing tissue was minced into small pieces using scissors. The tissue was digested in HG-DMEM supplemented with 1mg/mL collagenase type I at 38.5 °C for 8 hours. After incubation, collagenase was inactivated by diluting 1:1 with HG-DMEM supplemented with 10% fetal calf serum (FCS). The digested suspension was filtered on an 80 µm strainer, centrifuged at 300 *g* for 10 minutes and washed twice in PBS. Before seeding, cells were counted using a Burker chamber with the Trypan Blue dye exclusion assay. WJ-cell cultures were established in HG-DMEM standard medium composed of 10% FCS, penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, 2 mM L-glutamine and 10 ng/mL EGF. Cultures were established at a density of $1x10^5$ cells/cm^2 in T75 culture flasks. The flasks were incubated at 38.5 °C with 5% CO_2 and 90% humidity. The medium was replaced after 72 hours to remove non-adherent cells and then replaced twice weekly until cells reached approximately 80% confluence. Cells were then detached with 0.05% trypsin-EDTA, counted, and redistributed into new culture flasks at a density of $1x10^4$ cells/cm^2 to maintain and expand the culture for ten passages (P).

2.4 Cryopreservation and thawing

Cells at P3 were cryopreserved. Briefly, the confluent cultures were treated with 0.05% trypsin-EDTA and washed by centrifugation (200*g* at 4 °C, for 5 minutes) with cell culture medium supplemented with 10% FCS to neutralize trypsin-EDTA. The cell pellet obtained was resuspended in pre-cooled (4 °C) cryopreservation media in 1-mL cryovials. The cryopreservation medium was 90% (v/v) FBS and 10% dimethyl sulfoxide. The cryovials were maintained at -80 °C overnight and then plunged into liquid nitrogen (-196 °C). After a minimum of one month of cryopreservation, the cells were thawed in a water bath at 37 °C. The cells were diluted in culture medium and centrifuged twice at 200*g* for 10 minutes. The cell pellet was resuspended in culture medium and plated in T25 culture flasks. Aliquots of these cells were kept to evaluate the cell viability using Burker hemocytometer chamber using the trypan blue dye exclusion method, under phase contrast microscopy (Nikon Eclipse Ti, Tokyo, Japan). After thawing, other aliquots were used for population doubling studies, or expanded until P3 to evaluate specific MSC marker expression and multipotent differentiation capacity in comparison to freshly isolated cells.

2.5 Proliferation rate and CFU-assay

Proliferation of MSCs was determined as previously reported [6]. Doubling time (DT) from P 1-10 was assessed by plating $9x10^3$ cells into six-well tissue culture plates. Every 4 days, cells were trypsinized, counted and reseeded at the same density. Mean DT was calculated from day 0 to day 4. The DT value was obtained for each P according to the formula DT=CT/CD, where CT represents the culture time and $CD=\log(N_c/N_o)/\log 2$ represents the number of cell generations (N_c represents the number of cells at confluence, N_o represents the number of seeded cells). Data representative of three independent experiments are reported.

To obtain the cell proliferation growth curve, cells at P0, P3 and P5 were seeded into six-well tissue culture dishes at a density of 1×10^3 cells/cm^2. Every 2 days, until 14 days of culture, one well out

of the six was trypsinized and cells were counted using the Trypan blue dye exclusion method with a Burker chamber.

Colony-forming unit (CFU) assays were performed at P0 on freshly isolated cells at different densities (100, 250, 500 and 1000 cells/cm^2). Cells were plated in six-well plates for two weeks in HG-DMEM standard medium. Then, colonies were fixed for 1 hour with 4% formalin and stained with 1% methylene blue for 15 minutes in 10 mM borate buffer at room temperature. Colonies formed by 16 to 20 nucleated cells were counted under a BX71 microscope (Olympus Italia, Srl, Milano, Italy).

2.6 Osteogenic, adipogenic, chondrogenic and neurogenic cell differentiation

All the differentiation tests were performed when cells reached P3.

For osteogenic differentiation, cells were placed in plastic six-well plates at a density of 28 x10^3 cells per well. After the cells had adhered to the plastic, the inducer medium was added to the plate for 21 days and refreshed every three days. The medium was composed of HG-DMEM, 10% FCS, penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, 200 mM L-glutamine, 0.25 mM ascorbic acid, 10 mM β-Glycerophosphate, and 0.1 uM Dexamethasone [36]. Osteogenic differentiation was confirmed by positive staining of the extracellular calcium matrix using Von Kossa staining.

For adipogenic differentiation, cells were placed in plastic six-well plates at a density of 28 x10^3 cells per well. After the cells had adhered to the plastic, the inducer medium and the maintaining medium were added alternately, every 3 days for a total of 21 days. The inducer medium was composed of HG-DMEM, 10% FCS, penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, 200 mM L-glutamine, 0.1% insulin, 0.1 uM dexamethasone, and 1% indomethacin. The maintaining medium was composed of HG-DMEM, 10% FCS, penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, and 0.1% insulin [36]. Adipogenic differentiation was confirmed by positive staining of the lipid structures using Oil Red O staining.

For chondrogenic differentiation, cells were cultured in DMEM low-glucose, containing 100U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, 2 mM/l L-glutamine, 100 nM dexamethasone, 50 µg/mL L-ascorbic acid 2-phosphate, 1 mM sodium pyruvate (BDH, Atlanta, USA), 40 µg/mL proline, ITS (insulin 5 µg/mL, transferrin 5 µg/mL, selenous acid 5 ng/mL) and 5 ng/mL transforming growth factor-β3 (Peprovet, DBA Italia, 100-36E). Chondrogenic differentiation was assessed after incubating cells for up to 3 weeks [37]. Differentiation was evaluated by Alcian blue staining.

For neurogenic differentiation, cells were placed in plastic six-well plates at a density of 28 $x10^3$ cells per well. After the cells had adhered to the plastic, the pre-inducer medium was administrated to the plates for 1 day, followed by administration of inducer medium for 7 days. The pre-inducer medium was composed of HG-DMEM, 20% FCS, penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, and 0.0007% β-mercaptoethanol [15,38]. The inducer medium was composed of HG-DMEM, 2% FCS, penicillin (100 UI/mL)-streptomycin (100 µg/mL), 0.25 µg/mL amphotericin B, 1% DMSO, and 0.36 mg/mL BHA [39]. Neurogenic differentiation was confirmed by positive staining of the Nissl substance and granules, using Nissl staining.

For each differentiation experiment, a control group was performed by seeding cells at lower density ($9.5x10^3$) and feeding with HG-DMEM standard medium. These cells were stained using the same protocol as the treated cells. At the end of the differentiations, aliquots of non-stained cells were harvested and stored at -80 °C for further molecular analysis.

2.7 RNA extraction and Reverse Transcription-PCR analysis

Expression of specific MSC (*CD29*, *CD44*, *CD105*, *CD166*), pluripotent- (*Oct-4* and *c-Myc*) and hematopoietic (*CD34*, *CD14*, *CD45*) markers was investigated by RT-PCR analysis on fresh and thawed undifferentiated cells at P3. To evaluate whether cells could be well tolerated by the host once transplanted, expression of the Major Histocompatibility Complex, class I (*MHC-I*) and II

(*MHC-II*) was assessed. Total RNA was extracted from bovine WJ-derived cells using Trizol reagent (Invitrogen, Monza, Italy), followed by DNase treatment according to the manufacturer's specifications. RNA concentration and purity were measured using a NanoDrop ND1000 spectrophotometer (*NanoDrop* Technologies, Wilmington, DE, USA). cDNA was synthesized from 500 ng total RNA, using the iScript retrotranscription kit (Bio-Rad Laboratories, Hercules, CA, USA). Conventional PCR was performed in a 25 mL final volume with DreamTaq DNA Polymerase (Fermentas, St. Leon Rot, Germany) under the following conditions: initial denaturation at 95 °C for 2 minutes, 32 cycles at 95 °C for 30 seconds (denaturation), 55–63 °C for 30 seconds (annealing), 72 °C for 30 seconds (elongation) and final elongation at 72 °C for 10 minutes.

For differentiation experiments, total RNA was extracted from undifferentiated (control cells) and from induced WJ-derived cells and RT-PCR analysis was performed as described above. Bovine adult tissues (bone, fat, cartilage and spinal cord) were employed as positive controls for assessing the expression of *BGLAP, SPP1* and *SPARC* for osteogenic differentiation, peroxisome proliferator-activated receptor-gamma (*PPAR-y*), and leptin (*LEP*) for adipogenesis, collagen type 2α1 (*COL2A1*) and aggrecan (*ACAN*) for chondrogenesis, and glial fibrillary acidic protein (*GFAP*) and nestin (*NES*) for neurogenesis. Bovine-specific oligonucleotide primers were designed using open source PerlPrimer software v. 1.1.17, based on available NCBI *Bos taurus* sequences or on Mammal multi-aligned sequences. Primers were designed across an exon–exon junction in order to avoid DNA amplification. Primers were used at 300 nM final concentrations. Their sequences and the conditions used to amplify each gene are shown in Table 2. *GAPDH* was employed as a reference gene.

2.8 Flow cytometry

WJ-derived cells were analyzed by flow cytometry to determine the percentage of mesenchymal- (CD105, CD166), hematopoietic (CD34) and immunogenic (MHC-II) markers after isolation (P0).

For CD105, CD166 and CD34, primary mouse monoclonal antibodies and secondary antibodies rabbit anti-mouse FITC (Sigma, Milan, Italy) were used. For MHC-II, primary rat monoclonal antibody and secondary rabbit anti-rat FITC (Sigma) were used. Staining was performed as previously reported [40]. Cells ($1x10^6$ cells/mL) were labeled with primary antibodies in PBS with 3% of bovine serum albumin (BSA) (BDH; VWR International Ltd, Poole, UK) for 45 minutes at room temperature in the dark, followed by washing in cold PBS and a final incubation with secondary antibodies (1:50) for 30 minutes at room temperature in the dark. After incubation, cells were washed twice in ice-cold PBS and analyzed using a Millipore Guava easyCyte Single Sample Flow Cytometer. A minimum of 10,000 cells was acquired for each sample and analyzed in the FL1 channel.

The negative pattern was examined by applying the same cell suspension with the first incubation, and the result was included on the global compensation, in order to exclude auto fluorescence. A 488 nm filter was used in each analysis.

Off-line analyses of the flow cytometry standard (FCS) files were performed using Weasel software v.2.5 (http://en.bio-soft.net/other/WEASEL.htmL).

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Instat 3.00 for Windows (GraphPad Software, La Jolla, CA, USA). Three replicates were performed for each experiment (DT and CFU) and the results reported as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) for multiple comparisons by Student-Newman-Keuls multiple comparison tests was used. $P<0.05$ was considered as significant.

3. RESULTS

3.1 Cell morphology

The cellular yield was approximately $3x10^6$ cells per gram of minced WJ. The initial viability was

greater than 95%.

Cells were selected purely on their ability to adhere to plastic. Isolated cells readily attached to plastic culture dishes. Colonies started to appear within the first two days, reaching confluence after 5 days. WJ-derived cells were a morphologically homogeneous population of fibroblast-like cells in all passages of cell culture (Figure 1 A).

After thawing (at P3), viability was 80%. WJ-derived cells conserved their fibroblast-like shape.

3.2 Proliferation studies Cells WJ-derived cells were able to proliferate, reaching confluence in up to 10 passages.

At P1, the growth curve showed an initial lag phase of 3-4 days longer than that seen in the other passages and a subsequent log phase until 14 days. The WJ-derived cells showed more extensive proliferation at P5 but the intensity of proliferation was similar in all passages in the final culture days (Fig 1B).

DT remained constant until the seventh passage, then decreased significantly ($P<0.05$) until passage 10. The mean DT was 1.44 ± 0.24 days or 34.55 ± 6.33 hours (Fig 1C).

After thawing, the mean DT was 1.59 ± 0.06 days or 38.21 ± 1.68 hours (Fig 1C) with no statistical difference compared to fresh cells.

The number of cell colonies formed was counted at P0 on fresh cells. These cells were able to form an average of 1 CFU-F) (Fig 1D) for 221.68 ± 3.86 seeded cells. The highest number of colonies was found at the greatest density of seeding (Table 1).

3.3 Molecular characterization

As shown by RT-PCR, WJ-derived cells expressed MSC-markers (*CD29*, *CD44*, *CD105*, *CD166*) and lacked hematopoietic ones (*CD34* and *CD14*) from P0 to P10. *MHC-I* expression was present while *MHC-II* was not. Moreover, undifferentiated MSCs were found to express *Oct-4* and *c-Myc*, essential transcription factors for maintaining the primitive pluripotent state of embryonic stem

cells. After thawing, cells studied at P3 expressed the same MSC-mRNA markers such as *CD29, CD44, CD105, CD166, Oct-4, c-Myc* and *MHC-I*, as freshly isolated cells, but not *CD34, CD14* and *MHC-II*. (Figure 2A).

3.4 Flow cytometry analysis

Flow cytometry was used to evaluate the homogeneity of the cell population and to identify the subset of mesenchymal, hematopoietic and immunogenic cells. The cell populations tested were all $CD105^+$ and $CD166^+$, but negative for CD34 and MHC-II, as shown in Fig 2B.

3.5 Differentiation assay

The results of all differentiation assays are shown in Fig 3.

Osteogenic differentiation potential. After 21 days of induction, osteogenic differentiation was confirmed by von Kossa staining. The control (non-induced cells) was negative for von Kossa staining. RT-PCR analysis of *SPP1* and *SPARC* mRNA expression confirmed osteogenic induction but *BGLAP* was not expressed in induced cells.

Adipogenic differentiation potential. Cells were able to undergo adipogenic differentiation, as demonstrated by the development of positive staining for Oil Red O after 3 weeks of culture in adipogenic induction medium. Control cells, maintained in regular control medium, showed no lipid deposits. RT-PCR analysis of *PPAR-y* and *LEP* mRNA expression confirmed adipogenic induction.

Chondrogenic differentiation potential. Differentiation was identified by marked deposition of glycosaminoglycans in the matrix, stained with Alcian blue. The presence of COL2A1 and ACAN mRNA confirmed chondrogenic induction for this cell population.

Neurogenic differentiation potential. After 3 days of induction, neurogenic differentiation was confirmed by the morphology of the cells. The WJ-derived cells adopted the typical morphology of neural cells with dendrite-like processes. The presence of *GFAP* mRNA suggested that under these culture conditions, WJ-derived cells were induced to differentiate into glial cells. RT-PCR analysis

of bovine adult tissues (bone, fat, cartilage and spinal cord) showed expression of the specific studied genes.

The frozen-thawed cells were able to differentiate toward the same lineages as freshly isolated cells (data not shown).

4. DISCUSSION

This work allowed the isolation, characterization and differentiation of bovine stem cells derived from WJ. Our findings suggest that this tissue is a reliable source of presumptive stem cells, displaying intermediate features between adult and embryonic stem cells. These cells havewide potential clinical applications, because of their low immunogenicity and high differentiation potential. After digestion, large numbers of WJ-derived cells with > 95% viability (optimal value in terms of plating efficiency and cellular growth) were obtained. When cultured, these cells demonstrated strong adherence to plastic dishes and developed fibroblast-like morphology over time. Adherence is a fundamental property for the culture of stem cells [41]. The proliferation studies showed that WJ-derived cells reached high plating efficiency and had a high proliferation rate *in vitro* until P10, demonstrating a growth curve with a *lag* phase of few hours and an intensive *log* phase of 12 days. Moreover, the mean value of DT for 10 passages was 34.55 hours. During this intensive proliferation, the cells maintained their morphological characteristics. These data are in agreement with those obtained by other researchers who reported a high proliferation rate of human [37,42], equine [6], bovine [8] and feline [43] extra-fetal derived cells. It is very difficult to compare these data with those obtained from Cardoso et al. [32] because this study only reported the number of cells per mL found at different passages and the DT value was not calculated.

Bovine WJ-derived cells also showed the ability to produce clones. When seeded at different densities, they were able to form clones with a frequency that increased with the cell-seeding density, suggesting paracrine signaling between cells at P0 [44]. Moreover, WJ-derived cells showed a typical expression pattern expected for cultured stem cells [41] when analyzed by RT-

PCR. Indeed, these cells expressed a pattern of mesenchymal (*CD29, CD44, CD105, CD73, CD166*) and pluripotency (*Oct4, c-Myc*) genes with no expression of the hematopoietic *CD34*. The pluripotency genes are essential transcription factors for maintaining the primitive pluripotent state of embryonic stem cells. These data confirm the results obtained in equine, canine and bovine WJ [6,8,24,32,45-47] where pluripotent- and mesenchymal-associated markers were expressed.

For the first time, the expression of *MHC-I* and *MHC-II*, related to cell immunogenicity, was also evaluated to assess the usefulness of bovine WJ-derived cells for cell therapy. At each passage, these cells were negative for *MHC-II* and positive for *MHC-I*, consistent with findings of previous publications [6,8]. These findings reinforce the role of the extra-fetal tissue as an allogenic source for cell-based therapies in cattle. It is important to underline that RT-PCR alone is not useful for characterizing WJ-derived cells and that quantitative analyses are needed to make meaningful statements about their gene expression. Flow cytometry provides useful quantitative data on the percentage of cell reactivity. Indeed, we showed that >90% cells were positive for CD105 and CD73 while < 10% were positive for CD34 and MHC-II. These data confirm the mesenchymal nature of isolated cells, the lack of immunogenicity and underline the homogeneity of this cell line.

The capacity of MSCs to differentiate into a variety of cell types (adipocytes, osteocytes and chondrocytes) [48,49], has aroused interest in cell and gene therapy. Bovine WJ-derived cells, obtained by enzymatic digestion, were able to differentiate into osteocytes, adipocytes, chondrocytes and neuron-like cells in the same way as cells obtained by non-enzymatic digestion [32]. This suggests that these cells are capable of differentiation into multiple germ layers, an essential characteristic also observed in the pig [17], dog [24], horse [4] and chicken [50]. After 21 days of induction, mineral deposits were confirmed by Von Kossa staining and by the expression of *SPP1* and *SPARC* but not of *BGLAP*. This might be because *BGLAP* is expressed in terminally differentiated osteoblasts [51]. When stimulated to differentiate towards the adipogenic lineage, bovine WJ-derived cells were positive for Oil Red O staining and expressed genes involved in lipid biosynthesis and storage. mRNAs for *PPAR-γ*, crucial for the preadipocyte commitment [52], and

LEP, regarded as a late marker of adipocyte differentiation, were detected. The potential of bovine WJ-derived cells to undergo chondrogenesis was confirmed by positive Alcian blue staining and identification of markers commonly associated with the chondrocyte phenotype such as collagen type II and aggrecan, the most essential cartilage proteoglycan and key markers of chondrocyte differentiation [53]. *ACAN* expression was demonstrated, whereas a basal level of *COL2A1* was detected. The low expression of *COL2A1* might be related to the culture conditions in this study since chondrogenic differentiation of MSCs in monolayer culture appears to be dose-dependent and time-dependent in relation to the bioactive factors used [32,33]. *GFAP* and nestin, markers, expressed in neuronal precursor stem cells, have been detected in WJ-derived cells. The expression of both markers is probably related to the ability of these cells to differentiate either towards the glial cell lineage, as previously shown by Miki et al. [54] for 95% of cells isolated from human amnion, or toward neurogenic line as previously observed in pigs [55].

Whatever the reason, this cell line converted into a typical neuron-like morphology when appropriately induced.

Our findings suggest that, in agreement with reports of other researchers in several species [6,45-47,56], bovine WJ represents an alternative source of progenitor cells, that can be obtained by enzymatic methods for use in cell-based therapies. In our study, the digestion of tissue did not result in a reduced cellular viability or degradation of cellular surface receptors or alteration of cellular function as reported by Jeschke et al. [57].

Moreover, after thawing, the cryopreserved cells had a high level of viability (80%) and could be successfully expanded and differentiated. This demonstrates that bovine umbilical cord matrix cells can tolerate freezing without loss of functional integrity in terms of morphology, presence of specific stemness markers and differentiation potential, although renewal capacity was slightly lower than that observed in freshly isolated cells.

In conclusion, these data confirm that bovine WJ contain a niche of MSCs. However, further investigation, including pre-clinical studies and further study of immunological properties, are

needed to better understand their role in cellular therapy before *in vivo* applications of WJ-derived cells are considered. To date, there is only a single literature report of transplantation of caprine WJ-derived cells for wound healing, which showed promising effects [23]. The findings of this study reinforce the emerging importance of extra-embryonic tissues for derivation of cells that may be ideal tools in veterinary regenerative medicine.

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DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the reported research.

CONTRIBUTORS

ALC designed the study, performed isolation cells, acquired, analyzed and interpreted data, and wrote the article

CP performed proliferation study, molecular characterization and approved the final version of manuscript

AB performed cytofluorimetric analyses and approved the final version of manuscript

PE performed differentiation study and approved the final version of manuscript

FC designed the study, analyzed and interpreted data and revised the manuscript

LV conceived and designed the study, collected the umbilical cords, analyzed and interpreted data, and wrote the article

633 **Table 1.** CFU assay

634

Density cells/cm^2	Total cells	CFU	1 CFU each
100	950	1.5±0.71^a	633.33
250	2375	22.33±5.17^b	106.36
500	4750	57.92±4.31^c	82.01
1000	9500	146.15±2.78^c	65.00

635 Different small letters superscripts (a,b,c) indicate statistically different comparisons (P<0.05) between cell densities in
636 the WJ group.
637

638 **Table 2.** Oligonucleotide sequences used for RT-PCR analysis.
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Markers	**Forward (5'→3')**	**Reverse (5'→3')**	**Annealing T**	**Product lenght**
Integrin β-1 (*CD29*)	GTTGGTTCTGCAGTTACGATCAG	AACCAAACCCAATTCGGAAGTC	52°C	203
CD44 antigen (*CD44*)	AACAGTAGGAGAAGGTGTGG	TCATGAACTGGTCTTGGGTC	61°C	166
Endoglin (*CD105*)	ACAAGTCTTGCAGAAACAGTC	GATGTCTGGAGAGTCAGCTC	61°C	182
ALCAM (*CD166*)	GTATTTATTGCCTTCAGGTCCT	TCTACCAGGGAGCATTTATAGTC	59°C	755
octamer-binding transcription factor 4 (*Oct-4*)	CACACTAGGATATACCCAGGC	GGAGATATGCAAGGCAGAGA	60°C	177
v-myc avian myelocytomatosis viral oncogene homolog *(c-Myc*)	GCGCCGCATTCGCGAAACTT	TGAGGGGCATCGCTGCAAGC	58°C	214
CD34 molecule (*CD34*)	CCTGAAGCTAAATGAGACCT	AACTTTCTGTCCTGTTGGTC	58°C	173
CD14 molecule (*CD14*)	TCCGAAGCCTGACTGGTCTA	TGTCGGCTCCCTTGAGAAAC	56°C	104
Major histocompatibility complex I (*MHC-I*)	GATCTCACTGACCTGGCA	CTGAGGAGGTTCCCATCTC	60°C	199
Major histocompatibility complex II (*MHC-II*)	CCTCGCTTGCCTGAATTTGC	ACAGGTGCCGACTGATGC	53°C	299
Bone Gamma-Carboxyglutamate (Gla) Protein *(BGLAP)*	TCGGGCAAAGGCGCAGCCTTC	GCAGGGCTGCAAGCTCTAGACG	55°C	231
Secreted Phosphoprotein 1 *(SPP1)*	CGCCGATCTAACGTTCAGAGTC	GACTCTCAATCAGATTGGAATGC	55°C	199
secreted protein acidic and rich in cysteine *(SPARC)*	CTGGTCACGCTGTACGAGAG	CGGTGTGAGACAGGTACCCGT	55°C	232
Leptin (*LEP*)	CAATGACATCTCACACACGCAG	CGGCCAGCAGGTGGAGAAG	55°C	212
Peroxisome Proliferator-activated Receptor (*PPAR*-y)	CGCACTGGAATTAGATGACAGC	CACAATCTGTCTGAGGTCTGTC	55°C	199
Collagen type 1, alpha I (*COL1A1*)	CGCGGATTTGTTGCTGCTTGC	AGGTCCCATCAGCCCCATTGGT	55°C	269
Aggrecan (*ACAN*)	CGCTGTCTCGCCAAGTGTATGG	CGGTTCAGGGATGCTGACACTC	60°C	175
Glial Fibrillary Acidic Protein (*GFAP*)	GGCACCTTGAGGCAGAAGCTC	CTCCTGGAGCTCCCGCACCT	60°C	195
Nestin (*NES*)	ACCACTGAGCAGTTCCAGCTGG	TTGCAGGTGTCTGCAGCCGT	55°C	187
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	ATGAGATCAAGAAGGTGGTG	CCAAATTCATTGTCGTACCAG	60°C	190

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649 **Figure Captions**

650 **Figure 1.** Cell characteristics. (A) Monolayer of WJ-derived cells (A); magnification 20X; scale
651 bar= 20 µm. (B) growth curve at passages 1 (P1), 5 (P5) and 10 (P10); (C) doubling times for both
652 fresh and thawed cells. (D) colony of WJ-derived cells for CFU study. Magnification: X 20; scale
653 bar= 20 µm.

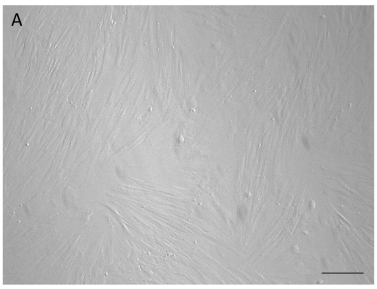
654

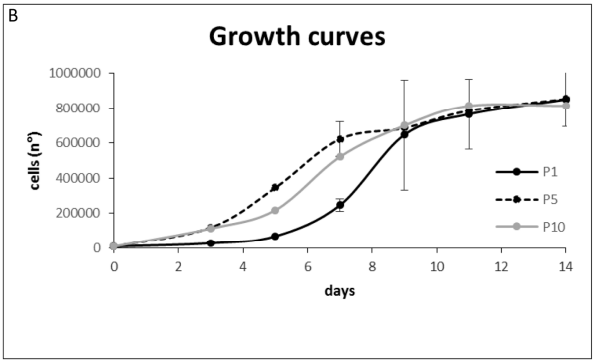
655 **Figure 2.** (A) RT–PCR analysis of mesenchymal (*CD29*, *CD44*, *CD105* and *CD166*), pluripotent
656 (*Oct-4* and *c-Myc*), hematopoietic (*CD34* and *CD14*) specific gene expression on WJ-derived fresh
657 and thawed cells. Major histocompatibility complex (*MHC*) I and II gene expression is also
658 reported. *GAPDH* was used as the reference gene. (B) Flow cytometry analysis of the expression of
659 mesenchymal (CD105 and CD166), hematopoietic (CD34) and immunogenic (MHCII) markers.
660 Histograms represent relative number of cells vs. fluorescence intensity (FL1). Black histograms
661 indicate background fluorescence intensity of cells labeled with isotype control antibodies only gray
662 histograms show positivity to the studied antibodies.

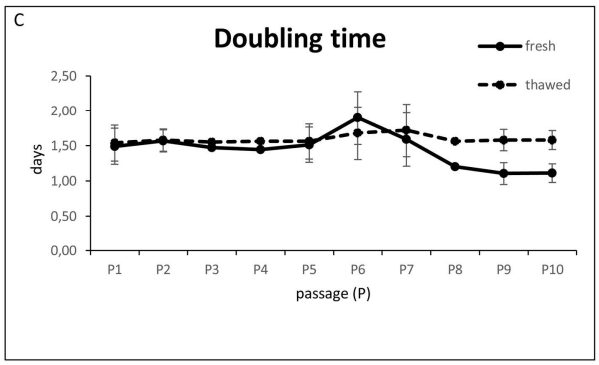
663

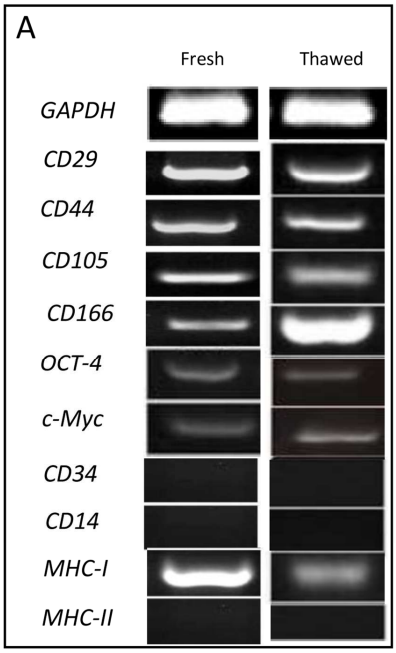
664 **Figure 3.** Staining of differentiated and control (undifferentiated) WJ-derived cells and respective
665 molecular expression. Osteogenic induced cells were evaluated for von Kossa staining and RT–PCR
666 analysis of *SPP1*, *SPARC* and *BGLAP*. Adipogenic induced cells were evaluated for Oil Red O-
667 stained cytoplasmic neutral lipids and RT–PCR of *PPAR-γ* and *LEP*. Chondrogenic induced cells
668 were evaluated for Alcian blue staining and RT–PCR of *COL2A1* and *ACAN*. Neurogenic induced
669 cells were evaluated for Nissl staining and RT–PCR of *NES* and *GFAP*; magnification: X 20; scale
670 bar=20 µm. *GAPDH* was employed as the reference gene. Bone, adipose tissue, cartilage and spinal
671 cord were used as positive controls.

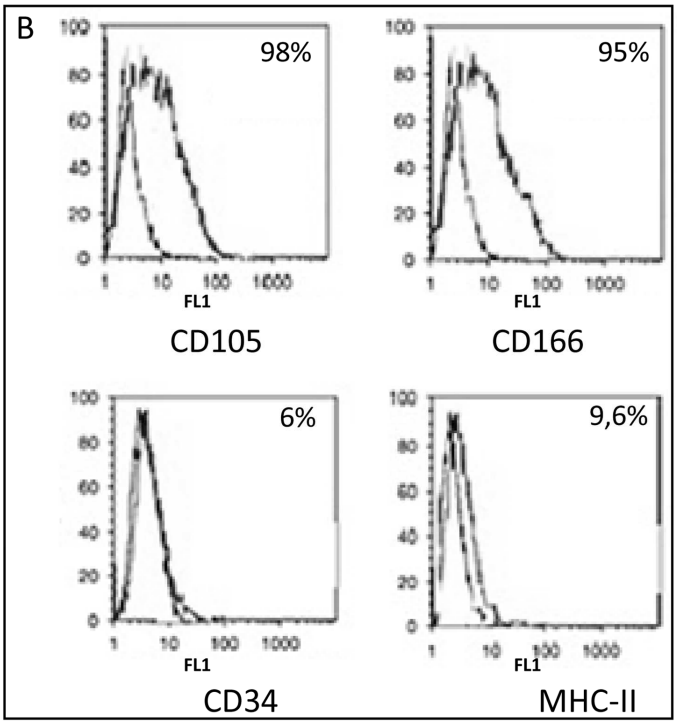
672

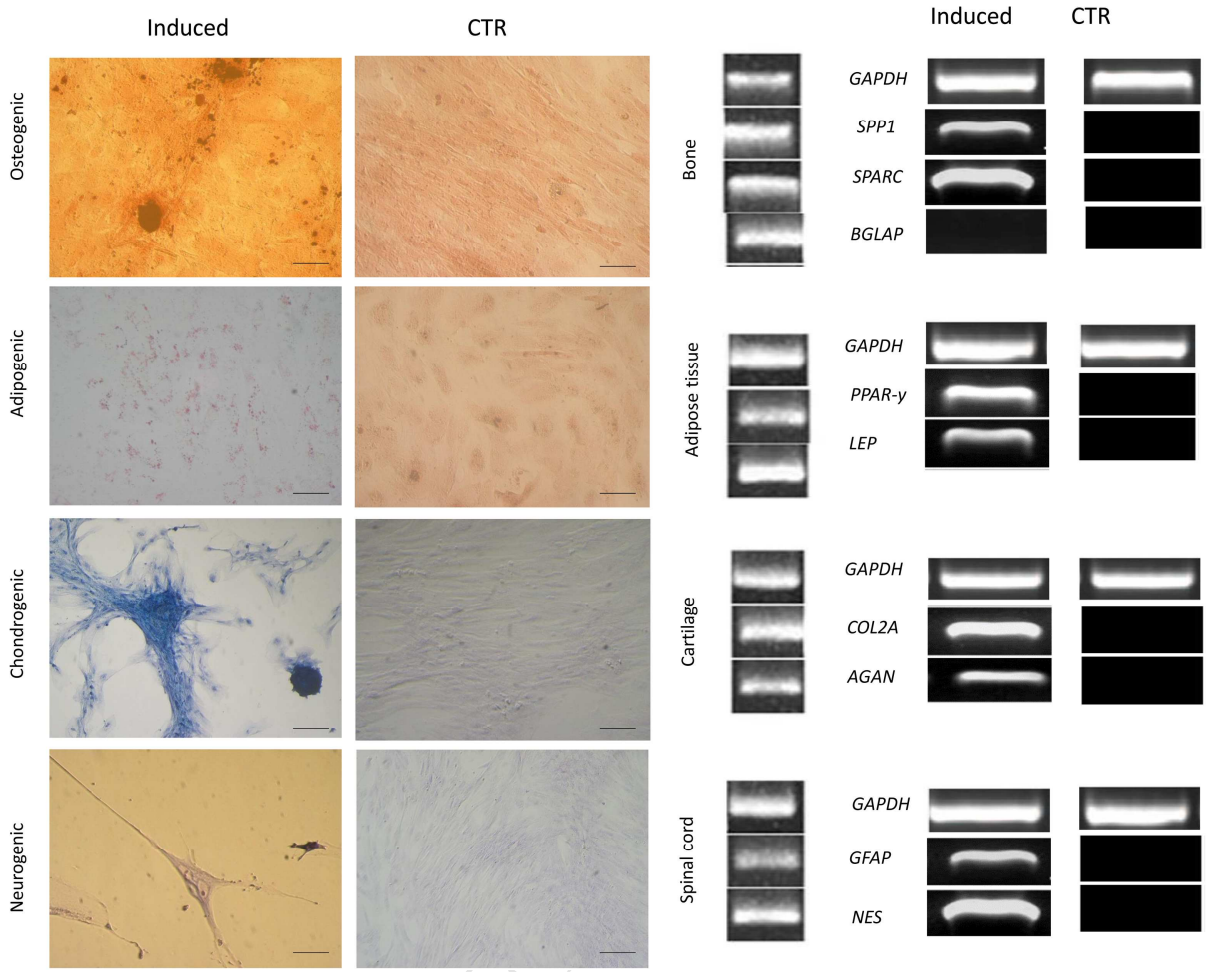












Bovine Wharton's jelly (WJ)-derived cells were isolated for the first time by enzymatic method

Molecular biology analyses revealed that these cells showed the CD antigen profile of MSCs

These cells possessed ability to differentiate in mesodermic and ectodermic lines

Their negativity to *MHC-II* reinforce the role of these cells as an allogenic source